ON SECONDARY COLONY DEVELOPMENT IN BACTERIA AND AN ANALOGY WITH TUMOUR PRODUCTION IN HIGHER FORMS

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I. INTRODUCTION

In a recent review (Haddow 1937) and in other papers (see Haddow 1936, Haddow and Robinson 1937) the author propounded the theory that the cancer cell represents a discontinuous and irreversible variant of the corresponding somatic cell, differing from the latter in the characters of fission-rate and metabolic behaviour. It was suggested that the problem of the origin of cancer might therefore be regarded as a special case in the origin of cellular variation, and suggestions were made as to the fundamental nature of the environmental changes leading to tumour production. In particular, it was postulated that these changes are frequently inhibitory in nature, and that the increase in growth-rate which marks the emergence of malignancy is due in many cases not to any primary *stimulation* of growth but represents the emancipation of potentially variable cells from chronic *inhibition*. Thus, evidence was presented to show that a number of the carcinogenic hydrocarbons exhibit growth-inhibitory properties not possessed by a considerable number of related non-carcinogenic substances. It was further found (Haddow, Scott and Scott 1937) that the inhibitory effect thus produced differed from that manifested by diverse toxic substances in its relative prolongation even after a single administration and in its primary independence of toxic action in the non-specific sense. The stages in the production of malignancy by the carcinogenic hydrocarbons (and possibly by other tumour-producing agents such as x- and gamma-radiation) were therefore conceived to be (1) the enforcement of a sustained retardation in the rate of growth of the treated normal cells; and (2), an irreversible dedifferentiation of the affected but viable cells accompanied by permanent metabolic alterations, increase in

growth-rate, and functional release from environmental inhibition.

It may be asked whether there is any other instance of the operation of such a principle of variation, and it is the object of the present paper to draw attention to such a case. In the author's opinion the environmental origin of organic change can nowhere be so clearly deciphered, or studied with such a degree of experimental control, as in the bacteria, and although the investigation of variation in bacteria possesses inherent disadvantages as compared with certain aspects of the same subject in other living forms, these are partly absent in the present example. The phenomenon of secondary colony formation has long been recognised by bacteriological workers, e. g., by Günther 1895, Hartmann 1903 in *Torula colliculosa* n. sp., Preisz 1904, and Eisenberg 1906. But it is almost certainly not familiar to the generality of investigators in other fields of biology, and it is clear that relatively little attention has been concentrated on its wider significance. It is for these reasons that the present account is submitted.

II. Secondary colony formation

The growth of a bacterial colony is in every case limited by the quantity of available foodstuff in the surrounding medium, and in certain cases it is also retarded by the local accumulation of inhibitory products of bacterial metabolism. Ordinarily therefore the increase of such a colony is brought to a close by the operation of these factors in differing degrees, the major influence being mostly a decrease in available nutriment to such a level as to render protoplasmic synthesis — and cell-division — no longer possible. In normal circumstances no further colonial change of any significance takes place. It is however a matter of old-standing observation that for certain bacterial strains the colony evolution is more complex than the above, since in these cases development is accompanied or followed by the appearance of so-called papillae or secondary colonies (daughter colonies, Knöpfe), which apparently arise from the substance of the primary or mother colony. It is, further, of the greatest interest that the cells of these secondary colonies can frequently be shown to metabolise some constituent of the medium which the cells of the primary colony are unable to attack. An important factor is obviously the inherent capacity for variation of any given strain, and it seems clear that " an organism which has no capacity for spontaneous variation to a carbon compound is not caused to vary to it by cultivation in contact with the compound no matter how long the contact may be prolonged " (Lewis 1934). It is nevertheless probable that daughter colonies may be observed at times in cultures of all species of bacteria, a view which was supported by Enderlein.

Klotz (1906) showed that a non-lactose-fermenting organism *B. perturbans*, if grown on solid media containing lactose, produced colonies which developed lactose-fermenting papillae. A further and important example of this phenomenon was provided in a strain of *B. coli* studied by Neisser (1906) and Massini (1907) and referred to by them as *Bact. coli mutabile*. When this organism was grown on solid media containing lactose and a suitable indicator such as neutral red, it formed primary colonies which were unable to split the available lactose and which therefore remained pale. During development, however, secondary colonies appeared which frequently possessed the property of fermenting lactose. The work of Massini was repeated by Burk (1908), and the succeeding years saw a number of papers confirming and extending these observations. Thus Müller (1909, 1911; see also Benecke 1909) described the production of papillae by certain strains of coliform bacilli grown in the presence of arabinose and by *B. typhosus* when grown on rhamnose or iso-dulcite. Similary, Burri and Duggeli (1909) and Burri (1910) described a coliform bacillus (*Bact. imperfectum*) not fermenting saccharose but yielding a saccharose-fermenting race (*B. perfectum*) after growth in medium containing this sugar. Other studies at that time included those by Kowalenko (1910) with single-cell technique, Jacobsen (1910), Thaysen (1911), Penfold (1911, 1912), Bernhardt and Markoff (1912), Mandelbaum (1912), Dobell (1912-13) and Baerthlein (1913), while among more recent contributions may be mentioned those of Stewart (1926, 1927, 1928), Haag (1927), Hadley (1927), Ramchandani (1929), Kennedy, Cummings and Morrow (1932), Lewis (1933, 1934), Kritschewski and Ponomarewa (1934), Hall (1935), Dulaney and Michelson (1935) and Deere, Dulaney and Michelson (1936).

Lewis (1933) classified his variable strains

— of *B. mycoides* — in three groups as follows : (1) those obtaining carbon principally from nitrogen-containing compounds but capable of variation in relation to sucrose; (2) those giving secondary colonies on agar containing protein alone, variation apparently being related to some unused fraction of the split protein; (3) strains giving secondary growth both in the presence and absence of sucrose but varying to sucrose in preference to protein. The addition of non-utilisable carbon compounds in respect of which the organism lacked the capacity to vary (organic acids as well as carbohydrates and alcohols) was without effect in all groups, nitrogen metabolism proceeding unhindered. The same author (1934) used eleven strains of colon bacteria variable to lactose or sucrose, two strains of *B. paratyphi B* variable to raffinose, and three strains of *B. typhi* variable to rhamnose, and his work is of special importance as showing that secondary colony formation may occur in synthetic media containing lactose as the sole source of carbon. According to Hall (1935) — in *Bact. coli mutabile* — " the ' mutant ' type rarely, if ever when pure, reverts to the parent type, and, as a rule, is indistinguishable from it morphologically, culturally, or serologically, except by its more rapid fermentation of lactose, and by its inability to form characteristic multiple papillae or secondary colonies."

Figs. 1 and 2 show the appearance of secondary colony formation in strains of *B. paratyphosus* B in which the secondary variant possessed the property of fermenting lactose. Müller (1909) discovered that *B. paratyphosus* B (although not *B. aertrycke*) produces secondary colonies when grown in the presence of raffinose, and Figs. 3-14 trace the development of a single colony of another strain of the same organism on raffinose-neutral-red-bile-salt-agar.

III. Possible interpretations

1. *Contamination hypothesis.*

It is clear therefore that there is no doubt of the reality of the phenomenon as one affecting many strains of a considerable number of bacterial species. It must however be recognised that several interpretations are theoretically possible, and these may now be examined. It has been suggested in the first place that the appearances might depend on a simple *contamination* of the culture by the organism appearing in the papillae. There is however a considerable body of evidence to show that no ordinary form of the contamination hypothesis is sufficient to account for the facts observed. In particular it must be stressed that not infrequently the cells of the secondary colonies can only be shown to differ from those of the primary colony in one biological character, that of utilising a constituent of the medium which the primary is unable to attack. This at least tends to indicate genetic relationship between the two forms. Again, secondary colony formation in a suitable strain is very commonly found to occur in every colony in each of repeated platings, and possesses a definite relation in time — as will be shown — to the development of the primary colony. Further, admixture of discrete colonies of the two types is not ordinarily met with : hence purification from the alleged contaminant is not possible. It is therefore apparent that contamination in the usual sense is hardly an acceptable explanation.

2. *Primary selection hypothesis.*

Even when the close relationship of the two forms be admitted, there is however the alternative possibility that they co-exist in a natural mixture or a symbiotic relationship, and that preponderance of one over the other is brought about by certain selective factors in the environment. But any simple selection hypothesis is greatly weakened by the fact that the behaviour of strains such as *Bact. coli mutabile* as described above, remains unchanged even after long continued purification (as by repeated plating of broth cultures derived from single colonies). It is further disproved by the results obtained from single cell isolations (e. g., Kowalenko 1910; Penfold 1912, p. 215) which Lewis (1933) regarded as excluding selection in many cases.

The problem is not unlike that in the work of Stephenson and Stickland (1933), in which experiments were conducted to test whether the hydrogenlyases produced during the 20-hour period of a culture were the result of selection. It was shown that when formate is introduced into a growing culture of *Bact. coli* the enzyme appears in full strength before the lapse of a single generation, i. e., before any demonstrable increase in the total or viable count. In this as in the case under discussion, it seems that

the phenomenon is a transformative rather then a selective process, and that the cause is represented by environmental changes in the medium. In any discussion of the possible selective origin of secondary colony forms it must be remembered that these variants can almost invariably be shown to possess a metabolic advantage in that they are capable of utilising a constituent of the medium not utilisable by the primary form. If their origin were purely selective therefore one might hope to detect signs of a continuous utilisation of the substance in question (e. g., lactose) from the beginning of the culture. As will be seen subsequently in another connection, this probably does not occur. While evidence is thus strongly against simple or primary selection as an explanation of secondary colony formation, there is no possible doubt that once variation has occurred the altered environmental circumstances are such as must exert powerful selective forces, leading to a relative preponderance of the variant and in many cases to overgrowth of the primary colony.

2a. *Biochemical changes involved, in relation to primary selection.*

At this stage some attention must be devoted to the evidence which is available regarding the biochemical changes involved in secondary colony formation. In the first place it is obvious that one is not justified in assuming that carbohydrates are not utilised simply because the medium fails to become acid. This was pointed out by Merrill (1930) and, in addition, Jones, Orcutt and Little (1932), working with strains of atypical colon bacilli, concluded from quantitative determinations that lactose in their experiments was utilised from the beginning of culture, although the lactose broth remained alkaline for periods varying from 3 or 4 days to 2 or 3 weeks. It is a possibility therefore, as has been pointed out by several workers, that both the parent and variant strains of *Bact. coli mutabile* are able to utilise lactose and that the difference in their behaviour is due to a difference in type of sugar metabolism. In 1934 Lewis remarked " much has been written on the problem of whether the specific sugar or alcohol is attacked from the beginning of a culture or only after a period of contact with it, but no very definite experimental evidence is available and opinions differ. " In 1927 however Stewart had investigated the same problem by a method employing *B. coli communis* as an indicator of the presence of lactose. He found that before the emergence of the secondary form *Bact. coli mutabile* consumed either no lactose or only minute quantities, e.g., less than 0.0325 per cent. in four days. On the other hand the primary form varied to the secondary form in a discontinuous manner, leading to an abrupt and steep increase in sugar consumption. In 1936 moreover Deere, Dulaney and Michelson carried out experiments, using the official gravimetric method for lactose determination of the Association of Agricultural Chemists, to determine whether the primary form of *Bact. coli mutabile* utilises lactose from the beginning of growth or only after the appearance of the secondary form. The results showed that the parent form of the organism uses only very small quantities of lactose, if any, before the secondary variant can be detected. On the other hand, the primary form showed similar ammonia production and pH changes whether grown in plain broth or lactose broth, indicating that it uses nitrogenous compounds as its source of energy. Further, the primary and secondary forms when grown in plain broth produced like changes in ammonia concentration and pH. These authors pointed out however that their results did not eliminate the possibility that minute amounts of lactose might be utilised by *Bact. coli mutabile*, since the lactose determination was not sufficiently accurate to estimate the disappearance of traces from a concentration of one per cent. It is of interest that for *E. coli* Hershey and Bronfenbrenner (1936) found no indication of a lactose-fermenting mechanism other than preliminary hydrolysis to the constituent monosaccharides. Real changes in lactose fermentation may be attributed to variation in the cellular activity of lactase, an enzyme which Hershey and Bronfenbrenner found to be intracellular and not liberated to any appreciable extent after death and autolysis of the bacteria.

3. *Transformation and secondary selection.*

Quite apart from the simple selection hypothesis already considered, the view was put forward by Smith (1913) and others that variation occurs in the absence of any specific sugar and that the sugar when present merely acts as a selective agent favouring the " spontaneous " variant. Stewart (1927) however thought there

was no basis for any suggestion that variation of this type is constantly occurring without the appropriate stimulus. As has already been mentioned, there seems little doubt that selection must operate immediately transformation has taken place, so that the next phase of the problem concerns the relation of the sugar to the *induction* of variation. Writing in 1933 Lewis thought there could be little or no difference of opinion that enhancement of fermentative power takes place only when the organism is cultivated in contact with the specific compound with which the variation is associated... " ability to attack unused nutrients is acquired through variation due to the specific stimulus exerted by the substance concerned. " But this author later modified his opinion (1934), and described experiments which he claimed showed that variation in certain coliform strains occurred in the absence of lactose, the variant cells being however so few as to escape detection by conventional methods of plating. He then regarded the evidence as conclusive " that variation of *mutabile* strains to a given specific carbon compound occurs spontaneously in media from which the compound is absent ", and looked on sugars and alcohols as acting as specific selective agents rather than as stimulators of variation. While Lewis still correlated the occurrence of variation with limitation of growth due to depletion of the medium, he felt himself obliged to abandon the theory of a specific inciting stimulus to variation in *mutabile* strains of bacteria. In the writer's opinion this problem of the relation between the carbon compound and the induction of variation is still not solved, and there appears to be a need for further critical experiments with purified synthetic media containing no trace of the substance in question.

3a. *Specificity of transformation.*

It is of interest that the study of training and adaptation in bacteria gives considerable support to the theory that primarily non-utilisable compounds in the substrate may influence the induction of variation in a more or less specific fashion. A good account of this subject is given by Knight (1936), according to whom " the enzymic constitution of bacteria and yeasts can change under the stimulus of changed composition of the media in which the cells are grown, and can respond to different substrates by production of the appropriate enzyme. " Among examples of this type of change may be quoted the work of Dienert (1900) who showed that a yeast usually unable to ferment galactose became able to do so after being grown in the presence of this sugar. Similarly Twort (1907; see also Penfold 1911, 1912) carried out important experiments on the induction of the property of lactose-fermentation (normally absent) in *B. typhosus*. Twort found that the long-continued propagation of this organism in a lactose-containing substrate might be followed by the appearance of variants capable of fermenting this sugar, and in addition he described a number of similar variations in carbohydrate fermentation when other organisms were grown for various periods of time in the presence of different sugars. Prolonged growth under such circumstances was thus shown to tend to the production of variants which fermented the particular substances with which the strain was cultured. Although this process was referred to as " training ", Penfold suggested that the *modus operandi* of the sugar might again be largely selective. Penfold also studied the fermentation of dulcitol by *B. typhosus*. Late fermentation of dulcitol is however recognised as a frequent " normal " attribute of this organism, and this author found that of several different strains of *B. typhosus* grown in litmus-dulcitol-peptone-water some formed acid in one or two days, although the majority did not do so till the fourth or fifth day or even later. When such cultures were plated out daily on agar containing dulcitol and neutral red, the proportion of red colonies to white showed a gradual increase from the second to about the fifth day, reaching a maximum when the peptone water culture had become acid. Arkwright (1930) regarded the history of this variation as " a typical case of the transformation of a culture which is unable to ferment a certain sugar or alcohol into one capable of rapidly fermenting it. " In such a case the opportunities for the operation of natural selection are obvious in so far as a few individual bacteria capable of fermenting the alcohol are afforded an excess supply of available foodstuff as compared with the non-fermenters, a preferential situation which is likely to result in the overgrowth of the latter by the former. But the critical point in the underlying biology is once more the question of the precise mode of emergence of the fermenting strain, and in this connection it is of importance that in this

case also the white colonies of the original culture on dulcitol-neutral-red-agar in the course of development produced red papillae composed of active dulcite-fermenters.

Karstrom (1937) investigated the relation between the presence of specific carbohydrates in the substrate and the development of the corresponding carbohydrases in bacteria. As a result he classified the cell enzymes as (1) constitutive enzymes produced constantly and independently of the composition of the media in which the cells were grown; and (2) adaptive enzymes produced only in the presence of, and in response to, specific substrates in the culture medium. According to Virtanen (1934), " in general it seems that the metabolic enzymes proper are always constitutive, whereas the enzymes (especially hydrolases) which convert the nutrients into suitable form for the actual metabolic reactions may be adaptive ". The apparently adaptive production of hydrogenlyases by organisms of the colon group was examined by Stephenson and Stickland (1932, 1933) and Yudkin (1932). The last author discussed the alternative hypotheses of selection and adaptation as possible explanations of the phenomenon, and decided in favour of the latter. Similar evidence was provided by Stephenson and Stickland, who showed that while the addition of formate to *Bact. coli* growing in tryptic casein-digest medium produced no simple selective action in either direction, adaptive production of formic hydrogenlyase occurred more quickly than would be expected on a purely selective basis. In a later study of the formation of galactozymase by *Saccharomyces*, Stephenson and Yudkin (1936) further weakened the selection hypothesis by demonstrating that specific and adaptive enzyme production occurred in the complete absence of cell growth or multiplication. According to Knight (1936), " the training of an exacting strain to become non-exacting involves a change in the enzymic constitution of the organisms. An exacting strain which requires, for example, tryptophan as an essential nutrient, when it is trained and becomes non-exacting and able to use ammonia as sole source of nitrogen, must have acquired enzymes to enable it to synthesise tryptophan using ammonia as nitrogen source. In general, in the training of exacting strains to become non-exacting, enzymes required for the synthesis of protoplasm from the simpler nutrients have to be produced. The suggestion is, therefore, that the new enzymes are produced as a direct reaction to the chemical stimulus of the new nutrients, in the absence of the normal nutrients. " In a study by Gladstone (1937) of the nutrition and nitrogen requirements of *Staphylococcus aureus*, the great majority of strains grew well on media of known chemical composition which included 16 amino-acids. Initial differences in amino-acid requirements were found to exist among these strains, but as the organisms were adapted to utilise fewer amino-acids such differences tended to disappear. Finally, by a process of training, strains were produced which could grow on a medium from which all amino-acids were excluded, and in which the main source of nitrogen was ammonia.

It must be mentioned that in most cases of the adaptive production of enzymes by bacteria the alterations are of the nature of fluctuational modifications, withdrawal of the specific substrate leading to atrophy or disappearance of the appropriate enzyme system. But the information they yield is sufficient at least to suggest that similar specific relationships may exist between organism and substrate in the induction of irreversible and heritable changes. In the view of Hershey and Bronfenbrenner (1936) phenomena suggesting the specific elaboration of enzymes corresponding to a defined substance present in the medium indicate no more than the realisation of a potential and perhaps latent physiological capacity of the bacterial species in question. This may be compared with the earlier suggestion by Grey (1924) that the bacterial enzymes concerned in the decomposition of carbohydrates are few compared with the number of substances which may be attacked. Grey postulated five systems which in various combinations might cause the degradation of such substances : an enzyme removing hydrogen, an enzyme removing oxygen, and enzymes splitting off groups of 1, 2 and 3 carbon atoms respectively, the last three being interchangeable or at any rate of common origin.

IV. Theories concerning the mechanism involved in transformation

From the evidence detailed above it is reasonable to conclude that the phenomenon of secondary colony development involves biological transformation, and that the main role of

selection is confined to the period following emergence of the variant. It now becomes necessary to consider the various possibilities concerning the mechanism of change. " In order that two differentiated halves may be produced, some event must take place by which a chemical distinction between the two halves is effected. " (Bateson 1913). Morphological appearances alone are of little or no value in deciding this question. The component organisms of secondary colonies may present no apparent structural divergence from the normal, although the writer has frequently found evidence of pronounced disintegrative changes in such cells. In a study of impression films of the secondary colonies produced by *B. paratyphosus* B (Schottmüller) on raffinose-agar, Kritschewski and Ponomarewa (1934) described and depicted an extraordinary series of large-celled variants of spindle- and trypanosome-like morphology. But such changes are irregular in occurrence and difficult of interpretation, and the mechanism of variation can best be studied from physiological rather than morphological data.

1. *Hybridisation.*

Attention must first be directed to the hypothesis which posits a sexual process in bacteria and hybridisation as the source of such variants. While the study of variation in bacteria is undoubtedly subject to certain disadvantages, it has been widely assumed that since such cells divide as a rule by asexual binary fission with a complete absence of the fundamentally non-essential influence of sex and conjugation, variation by hybridisation can well be excluded. But although one must always entertain the possibility of an autogamous or even syngamous fusion in bacteria as the lowest order of sexuality, it is curious to find that the mere occurrence of discontinuous and heritable variation has been regarded as evidence of sexual reproduction in these forms, a view dismissed by Henrici (1928) as a quite unwarranted assumption. From time to time however certain authors have attempted to describe or interpret the phenomena of bacterial variation in the terms employed for higher forms. Thus in a study of diphasic variation in the Salmonella White (1925) thought there could be no doubt that specific and non-specific qualities were in general alternatives comparable almost to a pair of Mendelian allelomorphs. Stewart (1926, 1927, 1928) is however perhaps the only author to attempt a description of secondary colony formation in Mendelian terms. He chose the paracolon bacilli and the colon bacilli as examples of stable and true-breeding non-lactose-fermenters and lactose-fermenters respectively, with *Bact. coli mutabile* as an unstable non-lactose fermenting organism regularly giving off a fermenting daughter-race; and he brought forward evidence which he interpreted as showing that these three groups are Mendelian variants of one species, of which paracolon is the homozygous dominant, *mutabile* the heterozygote, and colon the homozygous recessive. Since the positive character for fermentation is recessive he regarded the dominant factor — for *Bact. coli mutabile* probably correctly as will be seen later — as an inhibitor, and claimed that while all three groups have the power of fermenting lactose, the dominant and heterozygote also carry a factor which inhibits this property. Representing the inhibitory factor as I, its absence as i, and the factor for lactose-fermentation as F, he described the constitution of the three groups as follows : paracolon IIFF, *Bact. coli mutabile* IiFF, colon iiFF. The regularly recurring variation of *mutabile* Stewart represented as Mendelian variation brought about by the segregating of allelomorphs in a heterozygote, IiFF dividing to IIFF, IiFF and iiFF. The most powerful objection to such a hypothesis is the fact that the IIFF form is not found among the descendants of the papillae of *mutabile* in culture, a difficulty which Stewart suggested might be explained by an overgrowth of this form by the IiFF form, or by the association of a lethal factor with the IIFF combination. In discussing the objection that hybridisation must depend on gametic union, Stewart pointed out the lack of positive evidence that bacteria do not in fact conjugate and the possibility that, even if they do not, a heterozygote may arise by mutation affecting one member only of an allelomorphic couple. As a result of his studies Stewart drew up the following as the laws governing variation in native heterozygous bacteria : " 1. A bacterium heterozygous in a given character (e. g., power of fermenting lactose), if exposed to the appropriate stimulus, will give descendants of two kinds : (a) heterozygous like the parent, (b) homozygous recessive (fermenting lactose). Since the positive character is recessive to the negative,

the formulae of the two must be written IiFF/L for the heterozygote, and iiFF/L for the recessive. The homozygous dominant, IIFF/L, a paracolon, has not been found among the offspring of native heterozygotes. 2. The homozygous recessive or red race breeds strictly true, even after many subcultures on media indifferent for the character concerned. 3. If a race is heterozygous for two or more characters (e. g., fermentation of lactose and dulcite), and if it is exposed to the stimulus appropriate for one character it will segregate in this character only. 4. Segregation will only take place in answer to a definite stimulus and in adaptation thereto. There is no basis for the suggestion that variation is constantly taking place without the appropriate stimulus, but that the varying forms only survive when selected by appropriate surroundings, and that in consequence the variation falsely appears adaptive. "

Stewart's hypothesis has not received any degree of support, although it represents a very necessary and complete examination of one of the more important possible mechanisms involved in this type of variation. Henrici (1934) however thought there was no justification for regarding these variations as evidence of sexual reproduction, and opinion is general that the facts can be interpreted on a simpler basis. According to Henrici " we have ample evidence that variations similar to those exhibited by bacteria occur in other organisms known not to exhibit sexual reproduction. We may observe variants arising in secondary colonies or in sectors, in cultures of moulds and yeasts and unicellular green algae. In moulds, little localised tufts of aerial mycelium differing from the parent type in colour or spore formation may be considered homologous with secondary colonies. Now when moulds and yeasts reproduce sexually they form typical sexual spores, ascospores or zygospores, which may be readily observed and recognised. Cultures of moulds and yeasts may be found to exhibit all of the types of variation encountered in colonies of bacteria, without exhibiting any sign of sexual spores. The author has observed a dissociation of the yeast *Torula pulcherrima* into red and white strains and into smooth and rough forms without any trace of ascospore formation. Moreover, it has been possible to demonstrate typical variations of this type in cultures of a fungus (one of the smut parasites) which was started from a single spore *known to be in the haploid state*. Here there could be but one sex in the culture, yet permanent variations occurred " It is of further interest that Erikson (1935), in a study of the aerobic streptothrices, and Sherman and Wing (1937), for the coli-aerogenes group, were unable to obtain any evidence of a sexual mode of reproduction.

2. *Segregation of characters in non-equational fission.*

Topley and Ayrton (1923-24) pointed out that if the variations of bacteria are to be interpreted on the basis of genetics, the facts may be fitted to the theory more readily if we assume that a *segregation* of characters has occurred during a non-equational cell-division, rather than assuming that they have resulted from a *re-combination* of characters in a sexual fusion of cells (see Henrici 1934). " The biological factors discussed must have a material basis, and the suggestion that the variations observed are due to an unequal distribution of the substances concerned, at the moment when division of the bacillus occurs, is so obvious that it may well be provisionally accepted as a working hypothesis. Such acceptance does not imply any specific view as regards the structure or mode of reproduction of the bacilli. We know that multiplication by binary fission occurs, and this form of growth gives ample opportunity for such a segregation as we have suggested."

Variation by non-equational division is probably best exemplified by the phenomenon of colonial sector-formation. It has long been observed that whole sectors of a colony may show obvious variation from the remainder in one or more of a number of characters (e. g., microscopic or gross structure, opacity or consistency; biochemical activity such as pigment production or specific fermentations). A general study of the subject was given by Nirula (1928) and a mention of sector-formation in Ascomycetes by Dodge (1936), while Punkari and Henrici (1935) described interesting variations in a chromogenic asporogenous yeast (*T. pulcherrima*) involving both secondary colony formation and sectoring. It is of some interest that the angulation of the sector, and the distance of its apex from the centre of the colony, give a measure of the time of emergence of the variant in relation to the growth of the colony as a whole. It is also striking that such

sector-formation in colonial organisms is the simplest possible example of modification within the " Woodger's cell-cone " of developmental biology (see Woodger 1931; and Needham 1936, Fig. 13 and p. 63). Fig. 15 illustrates the appearance of lactose fermenting sectors in a non-lactose-fermenting organism, and other examples have been observed by the writer from time to time. Thus when certain plate-cultures of *B. paratyphosus* B (Tidy) were examined under the Leitz Ultropak at a magnification of about 50 diameters and in conditions of dull illumination, it was found that the colonies, which under other conditions of magnification and illumination appeared perfectly uniform, were sharply divided into two types according to colour. While some were dull white, others possessed a delicate olive-green colour, and in addition a number of colonies showed remarkably beautiful sector-variation in these two colours. Such appearances were probably dependent on differences in the surface structure of the two colonial types.

It is found as a rule that the cells of the sector breed true, while the bulk of the colony gives rise to further colonies which may show sector-formation in turn. That is, the relation between primary cell and variant is the same as in the case of secondary colony formation. This physiological similarity is of interest in the study of secondary colonies themselves, since the possible influence of selection in the induction of these variants can be discounted much more readily in sector-formation, for obvious reasons, than in the case of secondary colony formation. It is also of importance that Nirula's studies on sectoring were carried out with isolated cells obtained by a modification of the Burri single-cell technique.

3. *Dedifferentiation, dissociation, adaptive reduction.*

It is probable from the relation between normal and variant strains both in secondary colony formation and in sectoring that the variant is derived from the parent by a process involving dedifferentiation or loss. Since the change results in the manifestation of a character (e. g., lactose-fermentation) previously apparently absent, it follows that the material lost must be regarded as an inhibitor of the reaction or enzyme-system concerned. Such changes are usually discussed under the heading of " bacterial dissociation ", a term however which Gardner (1935) regarded as inept, and to which he preferred " adaptive reduction ". If the change under discussion is indeed a metabolic dedifferentiation which permits the utilisation of an otherwise inaccessible substrate, it may be compared with the metabolic reversions described by Fildes (1934). " It appears possible to assume that when an organism adjusts itself to a simple substrate with which normally it cannot deal, it is because at one time in the history of the species it had been in the habit of using that substrate, that the process is a process of atavistic reversion ", (as in the synthetic adjustment of the typhoid bacillus in the presence of ammonia, with which it cannot normally deal, but absence of tryptophan, on which it normally is dependent).

It is important that more than one degree of dedifferentiation may be shown. Thus Stewart (1927) described a typhoid race " Bucknall " which achieved full dulcite-fermenting capacity by two discontinuous steps, each of which took place in papilla formation on dulcite. The race thus formed pale red papillae from which came white and pale red colonies. The pale red colonies in their turn formed dark red papillae, which gave pale red and dark red colonies on subculture. The white, pale red and dark red forms were quite distinct and remained so after growth on dulcite-free media. Stewart interpreted the phenomenon as due to the successive segregation of two inhibitory factors. Lewis (1934) also noted tertiary colony formation on continued ageing.

4. *Bacterial mutation.*

The question now arises whether the emergence of secondary growth can justifiably be referred to as bacterial mutation. Although the term has been freely employed in bacteriological literature, note must be taken of certain objections and qualifications to its use. Jollos was among the first to point out that the term "mutation", coined for sudden changes occurring in the forms of life with sexual reproduction (metaphyta and metazoa), is not applicable to asexual bacteria in which testing of the mutation process by crossing experiments is impossible. Again, in the opinion of Haldane (1933) a given difference between two somatic cell types cannot be proved or disproved, by genetical methods, to be due to chromosomal

aberration or gene mutation, since such cells do not reproduce sexually and it is only by sex-reproduction that the geneticist can distinguish nuclear from plasmatic changes. " The proof that a change is due to a gene mutation can only be given if the altered gene segregates from its normal allelomorph at meiosis according to Mendel's laws... " (Haldane (1934). But according to the same author (1932) " there is no reason to think that bacterial mutation is a phenomenon essentially different from mutation in higher organisms, and it is not even clear that it is commoner. "

Much of the difficulty is due to the use of the term without adequate definition, and confusion has arisen through insufficient appreciation of, or respect for, its technical use, with all its added implications derived from plant and animal genetics. Further, many geneticists appear disinclined to allow the word in any but its artificial or technical sense. The situation is exactly similar to that in the description of the malignant cell as a somatic mutation (see Haddow 1937). It may be noted on the other hand that a definition of mutation sufficiently wide to include micro-organisms (and somatic cells) was given by Gates (1915). According to Bernhardt (1915) the term " mutation " must be used in bacteriology in a sense other than that of de Vries, and as implying that the descendants show new heritable characters, regardless of the means by which their hereditary constitution has been influenced, or of the extent of the change.

Assuming that the conception of mutation is valid in the present example, the simpler mechanisms may briefly be reviewed. The possibility of mutation by factorial loss (and especially by the loss of an enzyme inhibitor in non-equational fission) has already been considered as a probable interpretation in some of the cases at least. It is naturally of especial importance in circumstances where dedifferentiation or degradation of various kinds (e. g., serological) have been experimentally demonstrated, and it permits less scope for reversibility than other explanations. But apart from the loss (or gain) of individual factors, variation may be due to the temporary inhibition of one by another, a conception referred to by Toenniessen (1921) as " Valenzwechsel der Erbfaktoren ". The changes occurring in the cell were also likened (by Gates) to the shift of a polygon of forces moving to a new position of stability. Lastly, according to Henrici (1934) the assumption of a tautomeric structure of the living protoplasm of bacteria may explain the changes occurring in the genetic elements by means of mutation, and the resultant permanent modification forms or mutants. Thus Gotschlich (1929) considered it possible that the genes behave in a manner similar to tautomeric forms which exist in equilibrium while their substitution forms are unequivocal and definite.

5. *The side-chain theory.*

Müller (1911) regarded the type of variation under discussion, and particularly that shown by *B. typhosus* in the presence of iso-dulcite, as an adaptation to a substance which otherwise inhibits growth. Further, he sought to interpret such changes on the basis of Ehrlich's side-chain theory. According to this view *B. typhosus* possesses receptors which anchor iso-dulcite : growth is therefore inhibited since the bacillus is not able to use these receptors for assimilation. There is considerable likelihood that this conception is fundamentally correct, and experimental evidence is available to support it. Thus Knight (1936) discussed the inhibition of growth in nitrifying bacteria (*Nitrosomonas*, *Nitrobacter*) produced by organic compounds such as glucose, urea and asparagine, with special reference to the work of Winogradsky on inhibition of growth and of Meyerhof on inhibition of respiration. In such experiments respiration and growth are inhibited in parallel, growth-inhibition resulting from a respiratory inhibition which is due in turn to displacement of the normal respiratory substrate (in this case ammonia or nitrite) or to a block of the respiratory catalyst (as by glucose when applied in the absence of normal substrate). Similarly, Sobotka, Holzman and Reiner (1936) found that non-utilisable pentoses, and particularly xylose, have a retarding effect on the rate of fermentation of hexoses by brewer's yeast, an effect due mostly to competitive diffusion. Müller's hypothesis, that certain non-utilisable compounds in the substrate may inhibit growth through interference with the assimilation of utilisable compounds, is therefore seen to be an entirely reasonable one. Assuming its correctness he postulated that the mutation consists in the emergence of a race capable of overcoming this receptor-block.

V. Cellular inhibition and the origin of discontinuous variation

1. *The general concept.*

There can be no doubt that the cellular changes under discussion are intimately bound up with antecedent or coincident changes in the bacterial environment. In this regard Müller's application of Ehrlich's hypothesis is of much greater interest than any of the theories already discussed, since it suggests both cause and mechanism, while the others are concerned with alternative mechanisms alone. The mere proof of the correctness of any one of these alternatives (e. g., simple segregation, sexual segregation, or the various possibilities included under bacterial mutation) might in no way elucidate the real problem, viz., the nature of the force which induces such change. To the writer, the side-chain hypothesis seems of particular interest as representing a special case of what may be suggested as a principle of wider application, namely, that cellular inhibition of various kinds and degrees is the initial process in the induction of dedifferentiating, irreversible and therefore heritable changes in asexual cells. The source of inhibition appears to be mainly if not exclusively environmental in origin, so that knowledge of the nature of the significant environmental alterations gives an opportunity to correlate these with the resultant biological change. In this connection Table I shows the development of raffinose-fermenting secondary colonies in *B. paratyphosus* B (Tidy) — on 1 per cent. raffinose-neutral-red-bile-salt-agar — in relation to the growth of the primary (five colonies). The aggregate of these data is presented in Fig. 16, and data based on Figs. 3-14 are shown graphically in Figs 17 and 18. Lemon (1933) suggested that the *maximum expansion rate* in a bacterial colony be indicated by plotting the actual increase in unit intervals of time. If for each period of time (T) the bacterial count or area is N_0, N_1, N_2,... at times t_0, t_1, t_2,..., the following formula is used :

$$\frac{(N_1-N_0)}{(t_1-t_0)\,\frac{1}{2}\,(T_1)}, \qquad \frac{(N_2-N_1)}{(t_2-t_1)\,\frac{1}{2}\,(T_2-T_1)+T_1},\ldots \text{ etc.,}$$

where the suffix of the denominator indicates the mid-point of the period during which the increase has occurred. Fig. 18 shows the advantage of this method in demonstrating the relation of the emergence of secondary colonies to inhibition of the growth of the primary colony.

For the successful induction of discontinuous and permanent variation in any cellular character two main factors thus appear to be necessary : (1) an environmental source of interference with, or inhibition of, the given character, of such a nature as to be long-continued but nevertheless admit of the survival of the majority of the affected cells; (2) an organism possessing the inherent capacity for variation in respect of the character in question. The general concept has been well expressed by Noel Paton (1926)... " If changes in environment lead to changes in the chemical processes in the living units they may so alter the conditions that continued existence is impossible, or may lead to adaptation to the chemical changes, provided that the modification is not detrimental to existence under the altered conditions. " In the theoretical case of an organism growing in a completely unimpeded manner in a medium to which it is ideally adapted, no variation of any kind will be expected to occur. Such a state is most nearly approached in the logarithmic phase of bacterial growth, and Winslow (1935) has shown that if one transfers a culture in this phase of growth to a fresh medium of the same kind, the cell characters remain strikingly constant. It has already been suggested that depletion of foodstuff is the most important factor in the inhibition of growth of a bacterial colony growing in a limited medium, and it is certainly significant that the influence of increased density of inoculation is to accelerate secondary colony formation and the effect of frequent subculture to postpone both papillation and variation. " If subcultures are made daily on to fresh media, the change which initiates papilla formation can be deferred indefinitely in the bacteria carried on, for variation does not appear in them even if the appropriate sugar is continuously present. " (Stewart 1927). Papillation may also be prevented by the addition of utilisable substrate : thus Lewis (1933) described a protein-sparing effect in an interesting strain of *B. mycoides* in which, although secondary colonies appeared on agar containing protein alone, the variation apparently being related to some unused fraction of the split protein, no secondary growth occured on the addition of sucrose.

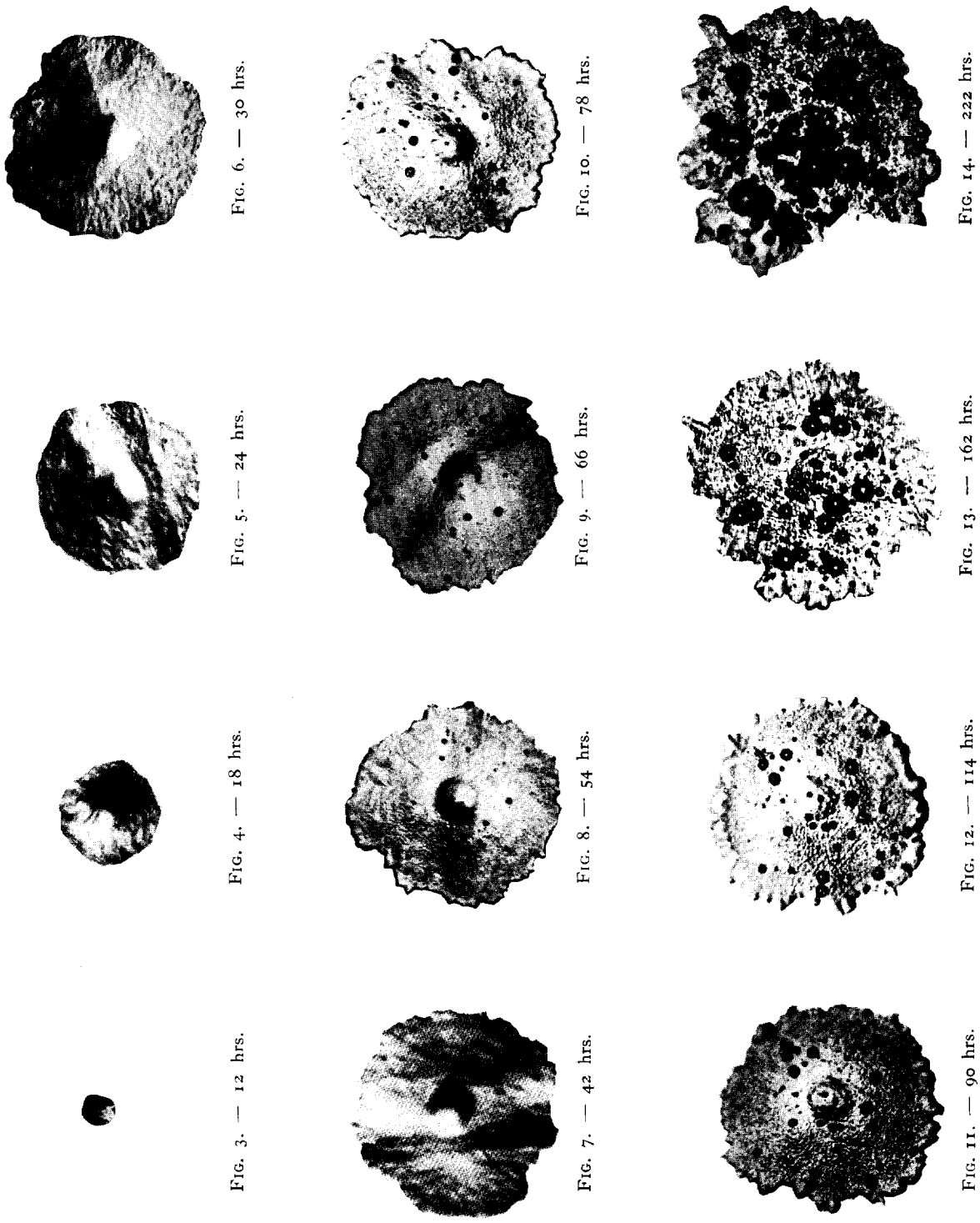

FIG. 3. — 12 hrs. FIG. 4. — 18 hrs. FIG. 5. — 24 hrs. FIG. 6. — 30 hrs.

FIG. 7. — 42 hrs. FIG. 8. — 54 hrs. FIG. 9. — 66 hrs. FIG. 10. — 78 hrs.

FIG. 11. — 90 hrs. FIG. 12. — 114 hrs. FIG. 13. — 162 hrs. FIG. 14. — 222 hrs.

FIGS. 3-14. The development of secondary colonies in *B. paratyphosus* B on neutral-red-bile-salt-raffinose-agar. Appearance of a single colony at 12, 18, 24, 30, 42, 54, 66, 78, 90, 114, 162 and 222 hours after inoculation. × 30.

2. *The nature of the inhibitory process and adaptive response.*

Speaking of the induction of heritable variation in general terms, it seems probable that identical end-results may be produced through inhibitory effects of different kinds. It may be regarded as certain that the gradual inhibition in growth-rate of a developing bacterial colony is not dependent on intrinsic causes, as was assumed by Stewart (1927; cf. Winslow 1935, above). The effect appears to be conditioned entirely by environmental changes, and the nature of these may now be discussed.

The physiological results of these changes constitute senescence. In relation to the induction of variation it is therefore important that secondary colony formation is closely dependent on the ageing of the primary colony, and that papillae appear first on the older or more central portions, where inhibitory changes are likely to be more intense. Inhibition of growth is almost certainly dependent on a metabolic inhibition, and it is of interest that Wooldridge, Knox and Glass (1936), in a study of the effect of age of culture on bacterial enzymes, found that the activity of most bacterial dehydrogenases appears to increase at first, to reach a maximum — usually within 24 hours — and subsequently to diminish. The same close relation between enzyme activity and phase of growth was further studied by Wooldridge and Glass (1937; see also the data given by Bach 1936 for decline in activity of various dehydrogenases in *B. proteus* in the first 96 hours of culture).

Lewis (1933) recognised the arrest of growth as due not to intrinsic but to extrinsic factors, the chief being depletion of nutrients. But he also described experiments to show that metabolic changes in the medium, quite apart from simple depletion, influence secondary colony formation. Lewis regarded it as improbable that any general law could be applied to all cases but thought that different extrinsic factors might cause arrest of growth in different organisms. In some cases for example retardation is probably assisted by alkaline changes in the medium — a circumstance which must imply an additional advantage to the acid-producing organisms of the secondary colonies — while in others inhibition is known to be due in part to accumulation of peroxides. Gause (1934) found that the growth of populations of *Saccharomyces cerevisiae* ceased before exhaustion of the energy sources of the medium, the effect being due to the establishment of a definite ratio between the concentrations of alcohol and sugar. Brown (1937) has recently discussed the production of growth-inhibiting substances by fungi. For a general account of the causes of inhibition of bacterial growth, whether by depletion or intoxication of the medium or by physical crowding, see Gay (1935).

In an interesting study of a luminous organism in relation to bacterial nutrition on agar Cruickshank (1934) investigated the question whether growth is limited by the accumulation of toxic substances or is the result of exhaustion of the nutrient substances of the medium. The activity of light production appeared to bear a direct relation to the state of growth (see also Harvey 1928, Shoup 1929 and Johnson 1936 on oxidation intensity and luminescence in bacteria. Harvey found exact agreement in the oxygen consumption of luminous bacteria as determined by the Thunberg microrespirometer and by the time which elapses before the luminescence of an emulsion of the bacteria in sea water begins to dim, when over 99 per cent. of the dissolved oxygen has been consumed). By using the light production of the colonies, recorded photographically, as a measure of metabolic activity, Cruickshank found that diffusible food substances are rapidly consumed from the agar in the neighbourhood and subsequently from a distance. It is of interest that the rate of diffusion of essential food substances was found to correspond approximately to that of haemoglobin in agar. Since the same principle appeared to apply in the case of other organisms, it was concluded that the important factor in determining the final amount of growth is the volume of agar from which the organisms may ultimately draw their food supply. Experiments were also described to show that whereas luminosity declined with exhaustion of the medium, it became restored following replenishment by agar patching. Cruickshank found it difficult therefore to explain his findings on the basis that arrest of growth is due to inhibition by metabolic products, but concluded that from the time that growth commences there is set up a gradient of concentration of nutritive material, and that with continued growth the concentration throughout the agar surrounding the colony

progressively falls. He recognised the important fact that although nutrition might fall below the level adequate for cell-division, it might still be sufficient for some measure of metabolic activity.

In a study of factors influencing bacterial populations Cleary, Beard and Clifton (1935) found that growth is first restrained by the changing ratio of total cells to available building material. This view was supported by Clifton, Cahen and Morrow (1936), who concluded that " growth-rate decreases as the concentration per cell of materials essential for growth decreases in cultures of bacteria in which a relatively high population has been established. " In a similar study Dambovi-ceanu and Roth (1936) also attributed arrest of development to disappearance of foodstuff, and were unable to detect the presence of inhibitory substances. From experiments described by Hershey and Bronfenbrenner (1937) it appears that under the usual conditions of cultivation, rates of growth and respiration of *Bact. coli* are limited by the rate at which oxygen can reach the cells, and that this limitation prevents rapid exhaustion of the nutrient materials. If however oxygen is available in excess, growth soon ceases as a result of oxidative removal of foodstuffs. They also stated that their work did not lend support to the view that accumulation of growth-inhibitory metabolic products is responsible for cessation of growth in bacterial cultures.

But although inhibition is very largely due to the negative influence of depletion or deprivation, there is no doubt that the same result may also be produced to some extent, as has already been indicated, by the positive effects of various harmful substances in the medium. These may be classified as (1) toxic substances foreign in nature, i. e., possessing no natural relation to the metabolism of the cells in question; (2) toxic products of metabolism (for the mathematical aspects of intoxication of a medium by catabolic substances see Kostitzin 1935, 1937); (3) potentially nutrient substances which are however not utilisable but cause inhibition by competitive interference with assimilation of the normal substrate. Similar to substances in class (3) are compounds which, while possessing high affinity for specific enzymes, cannot be attacked and therefore inhibit (e. g., the competitive inhibition of succinic dehydrogenase by malonic acid). And it is probably of related interest that in at least a number of cases (e. g., xanthine oxidase, Dixon and Thurlow 1924) the velocity of enzyme activity may be appreciably retarded by even moderate increase in the concentration of specific substrate.

Whereas acute inhibition or injury is followed either by death or by recovery, the type of mild yet chronic inhibition described in the present paper is such as does not cause death in any high proportion of the affected cells, at least for a considerable time. The conditions are probably similar to those in an ageing population of *Paramecium caudatum* (see Gause 1934, Fig. 5), where decline in reproduction-rate is accompanied by increasing environmental resistance, increase in the intensity of competition, and decrease in the capacity to realise potential growth. Secondly, gradual recovery is impossible on account of the uniform persistence of the inhibition. In the case of a bacterial culture allowed to age without renewal of medium or removal of inhibitory substances, it is obvious that inhibition must continue without remission, *provided the organism itself does not vary*. But the cell may undergo adaptive variation if such a process is inherently possible for any given strain, and there can be little doubt of the relative physiological advantages of such a change as is exemplified by the formation of secondary colonies. In the face of a condition of gradually increasing stasis we find the multicentric development of areas showing vigorous proliferation, and these proceed to increase both in size and in numbers at a time when the growth of the primary colony continues to be retarded or has altogether ceased.

Since an inhibition of the kind suggested may be produced either by the prolonged non-lethal action of certain " toxic " substances or by the constant presence of non-utilisable substances competing with the normal substrate, it is probable that the corresponding adaptive reactions are (1) a change whereby absorption of the toxic substance is lessened, the cell thereby becoming relatively refractory to its action; and (2) a change enabling the cell to metabolise the previously non-utilisable inhibitor and so emancipate itself from the inhibition due to this cause. Secondary colony formation is an ideal example of the latter mechanism, and it is of the greatest possible interest that proven examples of the former are also available. It

has long been recognised for instance that the exposure of pathogenic trypanosomes to non-curative concentrations of various trypanocidal drugs frequently leads to the appearance of new strains exhibiting a more or less specific drug-resistance (see Schlossberger and Schüffner 1934; Yorke and Murgatroyd 1935a, b; von Jancso and von Jancso 1935) and Ehrlich originally advanced the hypothesis that acquired resistance of protozoan strains is due to a loss of binding capacity. In an important paper Hawking (1937; see however Pedlow and Reiner 1935) investigated the absorption of arsenical compounds by normal and atoxyl-resistant trypanosomes by exposing the organisms *in vitro* at 37° C. to suitable concentrations of these compounds. The parasites were then centrifuged out and the distribution of the arsenical determined, either by measuring the trypanocidal activity of the supernatant fluid for fresh trypanosomes or by chemical estimations of the arsenic content of the supernatant fluid and of the deposited trypanosomes. Hawking found that while normal trypanosomes absorbed all the available drug from suitable concentrations of typical trivalent compounds (reduced tryparsamide, halarsol and novarsenobillon), living resistant trypanosomes absorbed little or none from similar concentrations of the same compounds, although absorption occurred if stronger concentrations were used or if the trypanosomes were dead. A similar difference in the behaviour of normal and resistant trypanosomes was observed when the parasites were exposed to reduced tryparsamide *in vivo*. Compounds to which the atoxyl-fast trypanosomes showed no resistance, e. g., phenyl-arsenoxide, sodium arsenite or tartar emetic, were absorbed to the same extent by both types of organism. It therefore appears that the comparative failure of atoxyl-fast trypanosomes to absorb typical trivalent arsenical compounds constitutes the explanation of the drug-resistance of these parasites. In a discussion of these results Hawking suggested their further interpretation on the hypothesis of receptor modification, the change being directed primarily against the side-chains on the benzene ring. Although drug-resistance in the trypanosomes is one of the best examples of adaptive transformation in the face of a dysgenic influence, there are others of considerable interest. Thus Neuschlosz (1919, 1920, quoted by Gay 1935) found quinine-fast paramecia capable of destroying 80 per cent. of the drug in solution while non-adapted organisms in the same solution produced a disappearance of less than 5 per cent. Again, according to Pett (1936, 1937) the effect of continuous subculture of yeast in cyanide indicates the possibility of developing a strain which will remain constant with or without cyanide.

The principles above defined are strikingly exemplified by the methods used to induce dedifferentiation — frequently irreversible or associated with renewed power of growth — in bacteria and protozoa generally. These include prolonged ageing, starvation, or the growth of an organism in its own antiserum or in suitable concentrations of bacteriostatic dyes, and are patently such as result in a profound interference with metabolism and growth. The mechanism of the bacteriostatic action of dyes was studied by Huddleson (1937) in the case of the Brucellae. "When a dye is added to culture media, a chemical or physical union occurs between the dye and certain food substances in the media necessary for bacterial growth. When united with certain dyes in the proper concentration, the food substances cannot be utilised for growth energy by Brucella. Thus, there follows no visible growth on the medium... The mechanism of dye bacteriostasis in the case of Brucella would appear to lie in inherent differences in the capacities of certain of the species to assimilate for growth energy the necessary food substances that are combined with certain dyes in the proper concentration." It is of interest that H → O changes similar to those produced by weak concentrations of antiseptics have also been described in cultures grown under conditions of deprivation, as in dilute broth or on agar with a diminished concentration of nutritive substances. The essential principle governing bacterial variation has been well expressed by Rettger and Gillespie (1935) on the basis of their observations of the process in *B. megatherium*. "The factors which stimulate cellular variation are apparently unfavourable to continued normal growth. When they accumulate very rapidly in a culture, development ceases before variation can take place. In other words, variation is possible only when favourable and unfavourable influences are so balanced as to permit slow growth in the face of untoward circumstances." It is also important that the natural occurrence of serological variations or

relapse strains in trypanosomiasis and in relapsing fever (see Cunningham, Theodore and Fraser 1934; Russell 1936) is probably to be interpreted as a response to the inhibitory properties of the serum which develop in the course of the infection. In a study of infections with *Trypanosoma lewisi* Taliaferro (1929; see also Coventry 1925) demonstrated the presence of a reaction product which inhibited the reproduction of the parasites without affecting their viability (see also Dingle 1936 on the growth-inhibitory powers of specific antisera).

VI. The analogy between secondary colony formation in bacteria and tumour production in higher forms

The appearances described above, and in particular the generation of a vigorously growing strain of cells in a senescent colony, seemed to the writer to present a striking resemblance to the emergence of a malignant variant in the cells of ageing animal tissues. Such a comparison raises the question as to how far one can be justified in any attempt to transfer principles governing the behaviour of bacterial cells to a study of the somatic cells of higher forms. In discussing another aspect of the same problem White (1926) wrote : " It is a very far cry from the genetic phenomena of the higher animals and plants to the serology of the Salmonella types. But when the same type of explanation, which has been found to hold good in the case of the former, is found readily applicable to the latter, criticism based simply on grounds of complexity is devoid of intrinsic force. " There is little doubt that certain cellular attributes are so fundamental that the principles which govern them must apply throughout the whole range of living matter. This seems most true of the laws pertaining to growth and its variations, and if so the change to which we allude as cancer in man and animals must be more basic than sexual differentiation itself. Again, it is a notable circumstance that various important functions in different forms, as for instance the fermentation of carbohydrate by yeast and sugar-utilisation by mammalian tissues, may show chemical similarity or even identity (see Kluyver 1932). It is probable then that certain laws, and especially those relating to growth, operate in a continuous fashion throughout the whole field of biology. As a further case may be chosen the similarity or identity (logistic law of Verhulst) of such varied examples as the sigmoid curve of growth of a bacterial colony in a restricted medium (Figs. 16, 17), the curve of human population growth (Pearl 1925), the curve of human body growth as estimated by increase in height (see Kostitzin 1937 p. 181), that of the growth of the blue whale as measured by increase in length (see Report on the progress of the Discovery Committee's investigations p. 18 : Colonial Office 1937), and the general statement that " populations of organisms of the most diverse kinds, ranging from bacteria and yeast to man, are found statistically to follow in their growth the logistic curve " (Pearl 1927).

If it be allowed that secondary colonies represent tumours of the primary colony, then we assume in the latter some element of unity or primitive organisation. It is interesting that Willmer (1935) postulated such colony-wholeness in tissue cultures of metazoan cells, drawing attention to " ...the suggestion that a tissue culture should not be regarded simply as a group of cells growing independently in a medium away from their natural position, but rather that it should be compared to a primitive organism, with a certain regulative capacity, that is to say, with a tendency to behave as a whole. " " It might be natural to suppose that the cells of a culture grow outwards into the medium because, by so doing, they move to areas of higher oxygen tension, lower CO_2 tension, and a weaker concentration of metabolites. This in a solid medium would tend to give the culture a spherical form, which in practice it does assume when embedded in the medium far away from any surface, and it would seem possible to imagine other fairly simple forces, such as those caused by the proximity of the glass surface of the coverslip or flask, which might tend to flatten the sphere into the disk shape characteristic of well-grown cultures; but the necessity for an explanation of the curious distribution of the layers of cells away from the glass and the evidence obtained from a study of the repair of injured cultures indicate that other more complex forces enter into the situation, and that the culture must be regarded not as a collection of individual cells but rather as a colony which shows evidence of polarity, and of the existence of definite axes. " A similar principle is probably represented, in its very simplest form, by the isolated bacterial colony. Conversely, the fundamental

nature of the cancer process is shown by the fact that, although the countless cells of the mammalian organism are constrained or aligned to play their part in the division of labour within the cell-state, they still retain sufficient individuality to enable them to shed their differentiation in the face of adverse local changes.

As the most constant and striking feature of cancer is rapid and continuous or uncontrolled growth of the malignant cells, so the outstanding characteristic of bacterial papilla formation is the more vigorous growth of the secondary variant. In both cases we have the apparent paradox that, in circumstances which lead to ageing or inhibition of the organism as a whole, there may appear a new cell type with a permanently increased rate of growth and, probably, with other physiological properties which enable it to resist, or to escape from, the increasing stranglehold brought about by environmental changes. It is important to emphasise that the alteration does not consist in the acquisition of a new function so much as in variation in the property of growth which is normally possessed by all cells excepting those of extreme differentiation. There is no doubt that different strains of malignant cells have genetically characteristic rates of growth, and these are probably the reflection of a similar condition in the corresponding normal somatic cells. Again the same principle probably applies for bacteria : thus Mason (1935), as the result of a comparison of the maximal growth rates and generation times of various bacteria under optimal conditions, looked on growth rate as often characteristic of the genus.

Aside from the general aspect, the argument may now be briefly recapitulated and the analogy continued in points of detail. It is a remarkable fact that the theories which from time to time have been evolved to deal with these phenomena are strikingly similar, or even identical, in their application. So we find that selection hypotheses (for selection in the origin of cancer see Fischer 1936, 1937), various aspects of the mutation theory, and even cellular hybridisation, have all been invoked in attempts to elucidate the origin of variations of this kind. Even from the beginning therefore it must be acknowledged that papilla-formation and tumour-formation have much in common, since they both represent a similar type of variation in an asexual cell. Again, it is clear that the cellular change in both cases is of the nature of a dedifferentiation which is mostly irreversible. It has been mentioned that variable bacteria may undergo more than one dedifferentiation, giving rise to secondary and tertiary colonies in succession, and similar changes have been shown to occur in the cancer cell. Thus Bittner (1931) showed that hereditary genetic changes might occur in the tumour cell during the process of transplantation, and that the cell might deviate from the genetic constitution of the individual which gave rise to it. Cloudman (1932) also demonstrated that a tumour in the course of propagation might change its character so as to become transplantable in a larger proportion of a mixed population, and found that in these circumstances one or more genes less were required for susceptibility in the host. Such changes are obviously in the direction of further dedifferentiation or change by loss.

As regards causation, it is the writer's belief that the origin of a malignant race of cells is not to be attributed to any process of direct stimulation of growth, as is so often assumed. From present knowledge it appears improbable that any primary stimulatory process will produce an increased amount of growth for any period of time much longer than the duration of its action. On the contrary, the increase in rate of growth of malignant tissue is maintained quite indefinitely, and long after it has escaped from the pathological environment, localised in time as well as space, in which it was engendered. When the application of a carcinogenic hydrocarbon is followed some months later by the appearance of a rapidly growing neoplasm, the result may suggest some process of direct stimulation. But such a view is not necessarily correct, and it seems less likely that the continued proliferation of malignant cells is due to any formative stimulus than that it represents the unmasking or release, in an adaptive dedifferentiation, of the capacity for growth which they always possessed, albeit in an inhibited sense, in their differentiated or integrated state. In the early history of cellular pathology a somewhat similar problem centred round the nature of inflammatory response, and according to Welch (1897; see Welch 1937), " the doctrine of Virchow was long accepted without question, that inflammatory cell-growth is the result of the action of external stimuli, the so-called inflammatory irritants, upon the cells, which are thereby directly incited to grow and multiply. The attack upon this

doctrine has been vigorously led by Weigert, who denies absolutely the power of any external agencies to stimulate directly cells to proliferation. He considers that to concede such a bioplastic power to external agents is equivalent to the acceptance of a kind of spontaneous generation of living matter. Weigert's views upon this subject have undoubtedly had a most powerful influence upon pathology. It has been such an influence as a good working hypothesis, whether finally demonstrated to be true or not, has often had in the development of science. In putting to the test of actual observation Weigert's hypothesis, we have been led to recognise the frequency and the importance of primary injuries to cells inflicted by external agencies. Not only various degenerations and necroses of entire cells, but more subtle and partial damage of cytoplasm and nucleus have been made the subject of special study. It has been recognised that our older methods of hardening tissues reveal often only very imperfectly the finer structure of cells, and new and better methods have been introduced which enable us to detect more delicate lesions of cell-substance which formerly escaped attention... Weigert's postulate of some primary injury to the tissues as the immediate effect of mechanical, chemical and other external agencies, which were formerly regarded as the direct stimuli of cell-growth and multiplication, has been fulfilled in many instances where such damage had previously been overlooked or unsuspected. It is his belief that in cases where we cannot now detect such primary injury more thorough search and better methods will enable us to do so."

With regard to malignant variation the present suggestion is that the adaptation is an irreversible one in response to chronic interfering changes in the environment of the type defined above, and it is a striking fact that agents such as x-rays and the gamma rays of radium, which the majority of workers now regard as inhibitory in their biological effects under all conditions, should rank next to the carcinogenic hydrocarbons in tumour-producing activity.

Pursuing still further the analogy between the formation of tumours and colonial papillae, attention must be directed to certain physiological similarities. First is the important circumstance that in both cases the variant cells show permanently altered metabolic properties which must confer distinct survival value and which probably furnish the additional energy demanded by the increased rate of growth. Secondly, both types of variation give indications of the importance of relative degrees of cell-stability (probably genetically determined) in the face of dysgenic changes. The significance of this conception has already been demonstrated for experimental carcinogenesis (e. g., Kreyberg 1936), and it is probably equally valid in the induction of mutation in bacteria. Thus the environmental changes which induce secondary colony formation in the case of certain cultures are quite without this effect in the great majority of bacterial strains, indicating the essential importance of cellular instability or an intrinsic capacity for variation of this particular type. Whereas adverse circumstances mostly produce a continuous cellular modification which is reversible in its earlier stages but later ends in death, the same changes produce in mutable strains a discontinuous alteration in cell characters with the emergence of a strain of increased relative viability.

Much discussion has centred round the question whether malignant change occurs initially in a single cell or in a number of cells almost simultaneously, and of considerable general interest is the fact that while secondary colony formation may affect but a single focus within the primary colony, it is more commonly multicentric in origin, and within a short time not infrequently affects several hundreds of foci in a colonial area of say 5-15 sq. mm. (Figs. 7-14, Table I). The outcome must obviously be the resultant of the regional distribution of inhibition and the degree of homogeneity of the affected bacterial population. Although inhibitory conditions at any given time must vary from point to point in the colony, and in general be more intense in the centre than at the periphery, the individual cells of the primary colony must exhibit a relatively high degree of biological uniformity. Assuming an essential similarity between bacterial papillae and tumours, and disregarding whether the exact analogy is between a single tumour and an individual papilla or the mass which may result from the fusion of several, it seems probable that while animal tumours may arise from a focus so small as to involve only a single cell, multicentric development is at least a possibility in cases where identical environmental conditions affect a number of biologically

comparable cells in a small volume of tissue.

It is of interest to find to what extent bacteriologists have been impressed by this general analogy. Massini himself acknowledged the resemblance (1907, p. 264 and Fig. 1) : in the words of Marie's abstract-review (1907)... " *à la façon d'une tumeur maligne*, ces granules traversent les différentes couches de chaque colonie ou bien se développent dans le voisinage du point ou celle-ci a offert trop de résistance. " While the present paper was in course of preparation the writer was interested to learn through Sir John Ledingham that Dr W. J. Penfold had many years ago been impressed by the resemblance between the formation of secondary colonies and tumours. In a subsequent correspondence Dr Penfold was kind enough to provide full details. Penfold took the analogy seriously as early as 1912 — when he first drew attention to the subject — and still later in 1922, when he published a paper (Penfold 1922) embodying his main views. After describing papilla-formation in *B. coli* when grown on monochloracetate-agar and in *B. typhosus* on iso-dulcite agar, he pointed out that the causative substances can generally be classified in two groups, potential food substances on the one hand, and growth-inhibitory poisons on the other. In view of the discussion of possible mechanisms of variation, it must be noted that Penfold was fully aware of the importance of the corresponding types of adaptive response, viz., acquisition of the capacity to metabolise a previously non-utilisable foodstuff, and, secondly, development of resistance to a poison to which the cells were formerly susceptible. Penfold apparently recognised the organisms of the secondary colonies as variants derived from the original bacteria, although in his interpretation he probably attributed undue primary importance to the role of selection. Further, he traced the fundamental resemblances between a bacterial colony and a composite tissue, and between the nodules appearing as secondary colonies in the one and as neoplasms in the other. Lastly, while interested mainly in extrinsic factors in the causation of tumours and bacterial papillae, he also indicated the significance of intrinsic (cellular) factors as influencing the variation process in each case. Penfold concluded with a short account of preliminary and projected experiments on the influence of aniline, toluidine, benzidine and naphthylamine on secondary colony formation in *B. coli*, *B. dysenteriae* Flexner and other species.

This concludes the discussion of evidence on which the general thesis, of biological analogy or even homology between secondary colonies and metazoan tumours, is based. It need hardly be pointed out that the basic phenomena of growth, senescence and variation can be studied with greater accuracy in the bacterial colony than in almost any other living material, and the developing colony is particularly suited to the study of the changes brought about by depletion and other inhibitory influences. Technical conditions are such as could scarcely be realised under any circumstances in the culture of differentiated tissues, and the main advantages are due to the high rates of growth and metabolism exhibited by many bacterial species growing in standard media, to the relative ease with which cultures can be manipulated in a pure condition, and to the facility with which the composition of the medium can be experimentally controlled.

TABLE I

Development of raffinose-fermenting secondary colonies in B. paratyphosus B in relation to the growth of the primary colony.

Hours after inoculation	Mean diameter of primary col. in micrometer units					Number of secondary colonies				
	col. 1	2	3	4	5	col. 1	2	3	4	5
17	9	10	8	10	5	0	0	0	0	0
24	20	20	21.5	19	16	0	0	0	0	0
28	26	25	24	25	23	0	0	0	0	0
40.5	37	35	34	41	40	0	0	0	0	0
48	43	40	40	47	46	0	0	0	0	0
52	42	40	40	50	50	?	?	?	0	?
65	43	42	40	58	55	11	18	20	11	2
72	43	42	40	60	63	14	27	20	12	4
78.5	43	43	40	65	65	22	30	25	14	6
91	48	50	46	75	74	32	48	39	35	33
100	50	45	45	78	70	45	52	39	41	47
118	50	48	45	78	93	65	79	68	91	76
137	50	50	50	80	85	100	102	115	148	108
160	53	52	50	100	95	95	134	115	160	130

VII. Summary

The development of our knowledge of secondary colony formation is summarised. Various possible interpretations are surveyed, and it is concluded that the phenomenon is usually an irreversible dedifferentiation and represents an

adaptation to unfavourable or inhibitory conditions. Inhibition may be due (1) to gradual depletion of the medium, (2) to the action of toxic substances in the environment, and (3) — possibly — to the competitive effect of non-utilisable substances in the substrate. It is suggested that the respective substances may impress some degree of specificity on the transformation in cases (2) and (3), and that selection must play an important part in case (3) once the variation has appeared. After a description of the nature of the adaptive response, attention is drawn to the analogy between secondary colony formation and tumour production in higher forms, and this is discussed in points of detail as well as on general grounds.

FIG. 1. × 30.

FIG. 2. × 10.

FIGS. 1 and 2. Secondary colony formation in strains of *B. paratyphosus* B on neutral-red-bile-salt-lactose-agar.

FIG. 15. Sector formation and appearances suggesting variation by non-equational fission in an organism growing on neutral-red-bile-salt-lactose-agar. Note lactose-fermenting sector at X. × 30.

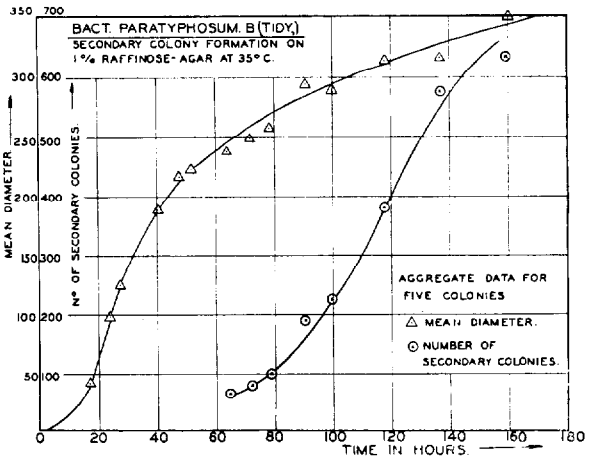

FIG. 16. Development of raffinose-fermenting secondary colonies in *B. paratyphosus* B in relation to the growth of the primary colony. Data from five colonies.

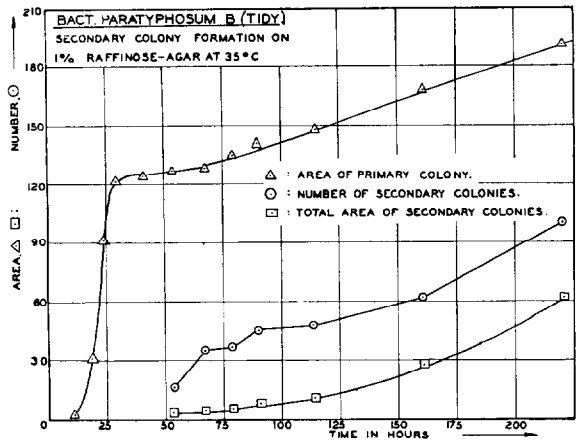

FIG. 17.—Development of raffinose-fermenting secondary colonies in *B. paratyphosus* B in relation to the growth of the primary colony. Data based on Figs. 3-14.

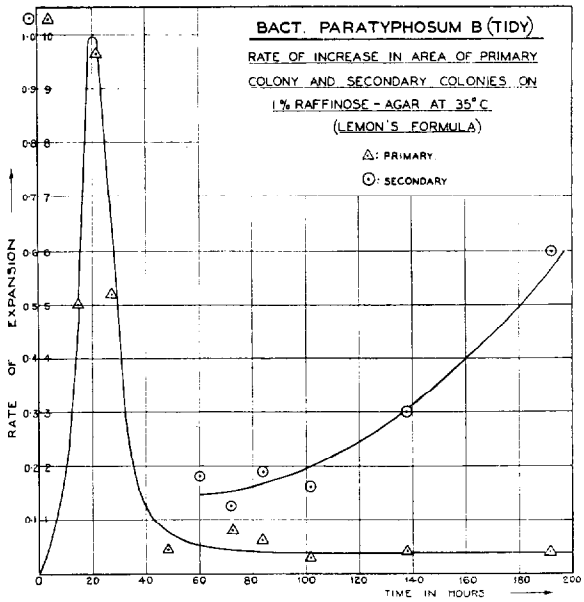

FIG. 18. Development of raffinose-fermenting secondary colonies in *B. paratyphosus* B in relation to the growth of the primary colony. Interpreted according to the formula of Lemon (1933).

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SUMMARY

The development of our knowledge of secondary colony formation is summarised. Various possible interpretations are surveyed, and it is concluded that the phenomenon is usually an irreversible dedifferentiation and represents an adaptation to unfavourable or inhibitory conditions. Inhibition may be due (1) to gradual depletion of the medium, (2) to the action of toxic substances in the environment, and (3)... possibly... to the competitive effect of non-utilisable substances in the substrate. It is suggested that the respective substances may impress some degree of specificity on the transformation in cases (2) and (3), and that selection must play an important part in case (3) once the variation has appeared. After a description of the nature of the adaptive response, attention is drawn to the analogy between secondary colony formation and tumor production in higher forms and this is discussed in points of detail as well as on general grounds.

ZUSAMMENFASSUNG

Der Autor setzt nacheinander die Entwicklung unserer Kenntnisse über die Entstehung der „sekundären Kolonieen" auseinander. Er zieht die verschiedenen möglichen Auffassungen in Betracht und schliesst, dass das Phaenomen allgemein betrachtet eine irreversible Entdifferenzierung ist und eine Anpassung an ungünstige und hemmende Bedingungen darstellt. Die Hemmung kann bedingt sein :

1. Durch eine stufenweise „Entleerung" des Milieu's.

2. Durch die Einwirkung von toxischen Substanzen auf die Umgebung.

3. — Vielleicht — durch die Mitwirkung von nicht brauchbaren Substanzen in dem Substrat.

Der Autor nimmt an, dass die entsprechenden Substanzen in gewissen Grade eine Spezifität der Umwandlung hervorrufen können (im Fall 2 u. 3) und dass die Selektion eine wichtige Rolle spielen müsse im Fall 3, wenn einmal die Variation eingetreten ist.

Nachdem er die Natur der Anpassungsreaktion beschrieben hat, richtet der Autor das Augenmerk auf die Analogie, welche zwischen der Bildung einer sekundären Kolonie und der Tumorenstehung bei den höheren Tieren besteht; er diskutiert ebenso die besonderen wie die allgemeinen Seiten des Problems.

RESUMEN

El Autor expone sucintamente el desarrollo de nuestros conocimientos tocante a la formación de colonias secundarias. Examina diversas interpretaciones posibles e infiere que el fenómeno es generalmente una desdiferenciación irreversible y representa una adaptación a condiciones desfavorables o inhibitorias.

La inhibición puede ser debida :

1. A la depleción gradual del medio.

2. A la acción de substancias tóxicas en los alrededores.

3. ... quizá... al efecto competitorio de substancias no utilizables en el substrato.

El Autor sostiene que las substancias respectivas pueden provocar algunos grados de especificidad en la transformación en los casos (2) y (3), en cuanto la variación ha aparecido.

Después de haber descrito la naturaleza de la respuesta de adaptación, el Autor llama la atención sobre la analogia que hay entre la formación de una colonia secundaria y la producción de tumor en los organismos superiores; discute tanto el aspecto general del problema como los detalles.

RÉSUMÉ

L'Auteur expose succinctement le développement de nos connaissances concernant la formation de colonies secondaires. Il envisage diverses interprétations possibles et conclut, que le phénomène est généralement une dédifférenciation irréversible et représente une adaptation à des conditions défavorables inhibitrices.

L'inhibition peut être due :

1. A la déplétion graduelle du milieu.

2. A l'action de substances toxiques aux alentours.

3. ... peut-être... à l'effet compétitif de substances non utilisables dans le substrat.

L'Auteur suggère que les substances respectives peuvent provoquer quelques degrés de spécificité sur la transformation dans les cas (2) et (3), et que la sélection doit jouer un rôle important dans le cas (3), une fois que la variation est apparue.

Après avoir décrit la nature de la réponse d'adaptation, l'Auteur, attire l'attention sur l'analogie qu'il y a entre la formation d'une colonie secondaire et la production de tumeur chez les organismes supérieurs; il discute aussi bien les détails que l'aspect général du problème.

RIASSUNTO

E' riassunto lo sviluppo delle nostre conoscenze sulla formazione di colonie secondarie. Sono prese in considerazione varie possibili interpetrazioni e si conclude che il fenomeno è, generalmente, uno sdifferenziamento irreversibile e rappresenta un adattamento a condizioni sfavorevoli o inibitrici.

L'inibizione può essere dovuta :

1. Allo esaurimento graduale del mezzo.

2. Ad azione di sostanze tossiche nell'ambiente circostante.

3. ... può darsi... ad effetto competitivo di sostanze non utilizzabili nel substrato.

Viene avanzata l'idea che le rispettive sostanze possano imprimere un certo grado di specificità nella trasformazione nel caso 2 e 3 e che la selezione deve avere una parte importante nel caso 3, poi che è avvenuta la variazione.

Dopo aver descritto la natura della risposta di adattamento, viene rivolta l'attenzione alla analogia tra formazione di colonie secondarie e produzione di tumori, e ciò viene discusso sia nei dettagli che da un punto di vista generale.

РЕЗЮМЕ

Автор кратко излагает наши знания об образовании вторичных колоний. Он рассматривает различные возможные об' яснения и приходит к заключению, что это явление представляет собою необратимую дедифференцировку, как ответ на неблагоприятные влияния, задерживающие рост.

Задерживающие влияния могут зависеть:

1. От постепенного истощения среды;

2. От действия токсических веществ в ближайшей окружности;

3. быть может: от конкурирующего влияния неусвояемых веществ в питательной среде.

Автор высказывает мысль, что соответствующие вещества могут действовать до известной степени специфично, вызывая трансформацию при условиях (2) и (3) и что естественный подбор играет важную роль в случае, (3) когда вариация уже появилась.

Описав сущность приспособительной реакции, автор указывает на аналогию, существующую между появлением вторичных колоний у одноклеточных существ и возникновением опухолей у высших животных.

Он останавливается как на общем значении этой проблемы, так и на ее деталях.